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Award Number: W81XWH-06-1-0466

TITLE: Modulation of the proliferation and metastasis of human breast tumor cells by SLUG (IDEA)

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REPORT DATE: April 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
29-04-2008	Annual	30 MAR 2007 - 29 MAR 2008

4. TITLE AND SUBTITLE
Modulation of the proliferation and metastasis of human breast tumor cells by SLUG (IDEA)

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0466

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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.

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Meharry Medical College
Nashville, TN 37208

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The objective of the project for the reporting period was to identify high affinity SLUG-regulated gene promoters from human breast cells. We over expressed 3xFLAG-tagged (C-terminal) human SLUG in the SLUG-negative MDA-MB-468 and MCF-7 cells through a lentiviral construct. Employing the ChIP-DSL techniques, we have identified 154 genes in the human breast cells that are tightly binding to the transcriptional silencer protein SLUG at the E2-boxes of their promoters.The gene promoters we are following up include those of claudin 7, VDR, UBE2D3 and dynactin 5. By mutational analysis, we identified two distinct motifs in the repressor domain of the SLUG protein as essential for the repressor function of SLUG. We further characterized the structure and function of the Pmotif of SLUG repressor domain as a unique CtBP1-recruiting site. We are planning to design peptide aptamer from the P-motif region of human SLUG protein and evaluate whether that aptamer can inhibit the repressor activity *in vitro* and *in vivo*.

15. SUBJECT TERMS
Breast cancer, SLUG, transcriptional silencing, molecular decoys, metastasis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	34	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

SLUG is a zinc finger transcriptional repressor protein that mediates its action through the binding to E2-box sequences (5′-CACCTG-3′) at the promoters of its target genes. SLUG and the other member of its family, SNAIL, are known to down regulate the expressions of many cell adhesion molecules (1-5). While SNAIL is reported to play a major role in these regulations in the non-breast cells (1-3), SLUG seems to be the major player in the human breast cells (4, 5). We have reported that SLUG also regulates the expression of the tumor suppressor protein BRCA2 and cytokeratins 8 and 19 in human breast cells (4, 5). An interesting aspect to add is that all the highly invasive human breast tumor cell lines express high levels of SLUG whereas the non-invasive breast cells are either SLUG negative or express very little of this protein. We postulated that a high level of SLUG protein in the breast epithelial cells inhibits the expression of BRCA2 thus promoting unfettered growth of the cells as well as the inhibition of the cell adhesion molecules by SLUG helps the proliferating tumor cells to transform to the mesenchymal cells and ultimately to metastasize. We have planned a series of experiments to **(i)** identify SLUG-target gene promoters to design high affinity SLUG-binding ds-DNA decoys; **(ii)** characterize the co-repressor binding domains of SLUG to design high affinity peptide aptamers that will block the binding of the co-repressors to the SLUG protein; and **(iii)** deliver the siRNA, ds-DNA decoy and peptide aptamers to human breast tumor cells to efficiently knock down the SLUG activity and to evaluate the effects of this ablation on the proliferation, invasiveness and metastasis of these cancer cells in 3D-tissue culture and mice models.

BODY

Task outlined in the approved Statement of Work for this period of the project

Task 1. **Identification and analysis of the promoters of SLUG-regulated genes to design high affinity SLUG-binding ds-DNA decoys (Months 1-15):**

Task 2. **Characterization of the co-repressor binding domains of SLUG to design high affinity peptide aptamers that will block the binding of the co-repressors to the SLUG protein (Months 16-27).**

Our progress/accomplishments associated with the task are as follows:

1. Evaluation of the SLUG-binding gene promoters in human breast cells by ChIP-DSL technique. This part of the work is a continuation of what we reported last year. We over expressed 3xFLAG-tagged hSLUG in SLUG-negative human breast cells like MDA-MB-468 and MCF-7 cells (Fig. 1). We employed the lentivirus-based tetracycline-inducible expression system (Invitrogen) for this purpose (6).

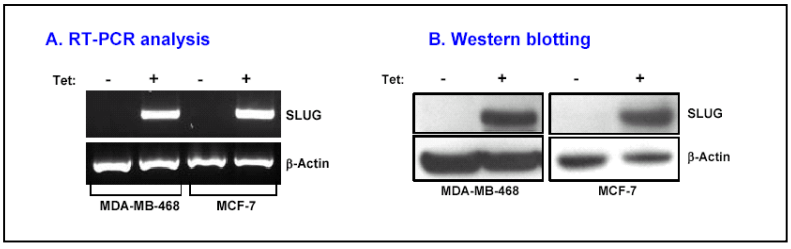


Fig. 1. RT-PCR (A) and Western blotting (B) analyses data showing tetracycline-inducible expression of hSLUG mRNA and protein, respectively. β-actin mRNA and protein were used as loading controls in these studies.

We evaluated the functionality of the C-terminally 3xFLAG-tagged SLUG in these cells using a *Renilla* luciferase-VDR promoter construct (6) in pRL-Null vector (Promega). The FLAG-tagged SLUG was functional in both of the recombinant cells in a tetracycline-inducible manner (Fig. 2). Using the reagents, promoter microarray chips and the protocols from Aviva Systems Biology (San Diego, CA) we analyzed 20,832 human gene promoters for their binding to SLUG (ChIP-DSL technique, Fig. 3). We used anti-FLAG monoclonal antibody (M2, Sigma) for this purpose. The experiments went well, as is documented by the array image (Fig. 4) and the scatter plot (Fig. 5). We repeated the procedures three times and identified 154 gene promoters as binding relatively tightly to SLUG.

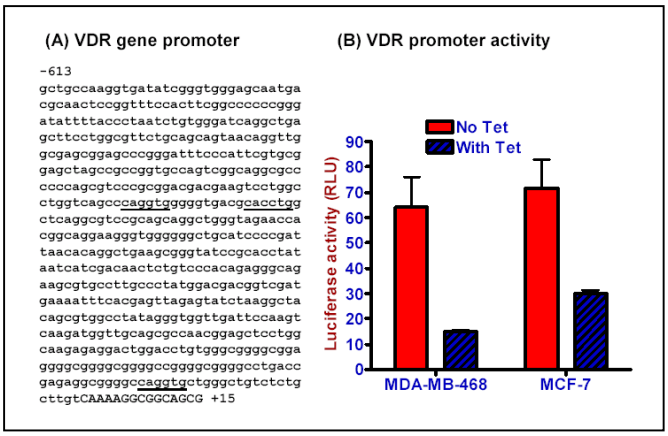


Fig. 2. Negative regulation of VDR gene promoter activity in SLUG-expressing human breast cells. (A) Nucleotide sequence of human VDR gene promoter showing (underscored) the SLUG-binding E2-box (CAGGTG/CACCTG) elements. The upstream sequences are shown in lower case letters. The 5′-end of the exon 1 sequences is in uppercase letters. (B) Dual luciferase assay (4) data showing the repression of the function of VDR gene promoter in the recombinant MCF7 and MDA-MB-468 cells. Results are mean ± SE (n=6). The differences in the luciferase activities between the control and the tetracycline-induced cells were statistically significant ($p<0.001$).

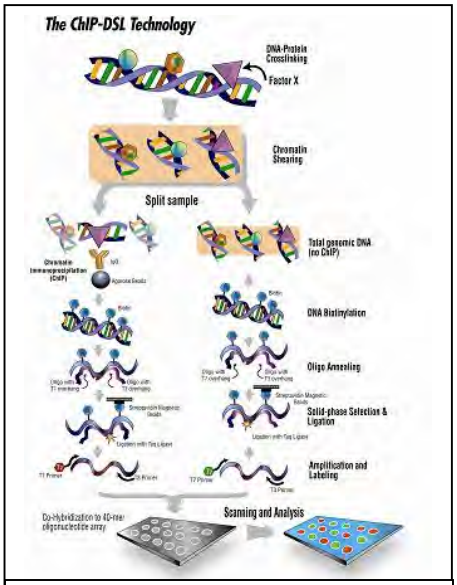
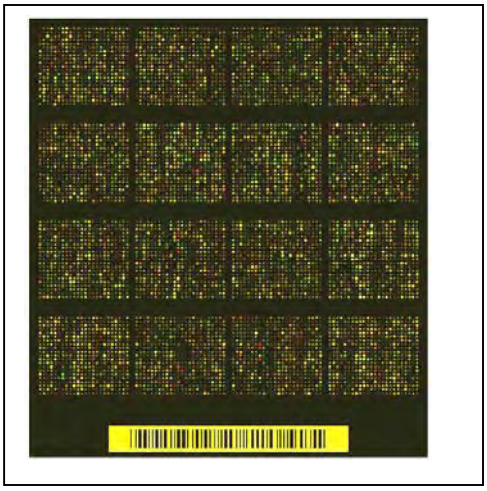


Fig. 3. The ChIP-DSL technique

These genes include those we reported previously last year. Other genes are being verified by independent ChIP analysis and RT-PCR analysis in the presence and absence of tetracycline (1 μg/ml) in the growth medium. The gene promoters we are following up include those of claudin 7, VDR, UBE2D3 and dynactin 5.

2. Down regulation of VDR in SLUG-expressing human breast cells. Since VDR and SLUG proteins are relevant in human breast cancer etiology, we characterized further the interactions of SLUG and the VDR gene promoter in the human breast cells. With cultured human breast cells, we found that there is an inverse relationship between SLUG and VDR gene expressions. The noninvasive MDA-MB-468 and MCF-7 cells do not express SLUG gene and they have significant levels of VDR mRNA and protein (Fig. 6A and 6B). Whereas, the highly invasive BT549 cells expresses SLUG but no VDR (Fig. 6A and 6B). When we induced the expression of SLUG in the recombinant MDA-MB-468 and MCF-7 cells, the levels of the VDR protein decreased significantly (Fig. 6C). Expression of non-functional SLUG protein did not cause any such effect on the VDR protein levels (data not shown). Our immunofluorescence microscopy data further verified the down regulation of VDR gene expression in the presence of SLUG in the recombinant MDA-MB-468

cells (Fig. 6D). These data strongly suggests that SLUG inhibits the expression of VDR gene in human breast cells.



3. Down regulation of UBE2D3 in SLUG-expressing human breast cells. Since UBE2D3 and SLUG proteins are relevant in human breast cancer etiology, we characterized further the interactions of SLUG and the *UBE2D3* gene promoter in the human breast cells. UBE2D3 is the E2 enzyme for the polyubiquitin tagging and final disposition by proteasomal degradation of P53 and cyclin D1 among others (7-10). When we induced the expression of SLUG in the recombinant MDA-MB-468 and MCF-7 cells, the levels of the UBE2D3 mRNA and protein decreased significantly (Fig. 7).

Fig. 4. Image of Chip-DSL promoter micro array/ZAR2 binding. Enriched/Input ratio, Chipped DNA: Red, Input DNA: Green. Looking for orange and red dots; Green dots: No binding; Yellow dots: No enriched binding (Ratio ~1.0).

Expression of non-functional SLUG protein did not cause any such effect on the VDR protein levels (data not shown). siRNA-mediated knock down of SLUG gene expression in the SLUG-expressing invasive MDA-MB-231 cells, up regulated the levels of the UBE2D3 protein (Fig. 8). These data strongly suggests that SLUG inhibits the expression of *UBE2D3* gene in human breast cells.

4. By mutational analysis, we identified two distinct motifs in the repressor domain of the SLUG protein as essential for the repressor function of SLUG. Human SLUG protein is 268 amino acids long with two functional domains: (i) the N-terminal repressor domain, and (ii) the C-terminal DNA binding domain (Fig. 9A and 9B). The DNA binding domain has five C_2H_2 type zinc fingers, which are essential for the interaction of this repressor protein with the E2-box sequences (CAGGTG/CACCTG) at the promoters of SLUG-target genes (1-3).

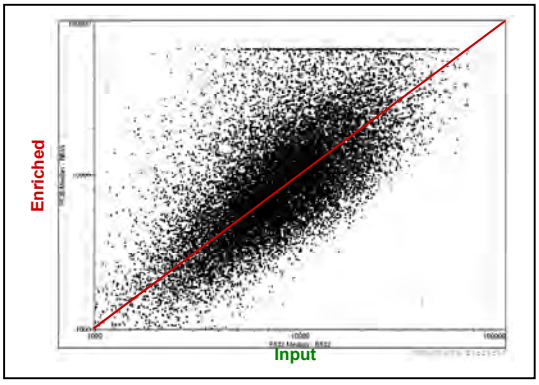


Fig. 5. The scatter plot showing the gene promoters that binds to SLUG in MCF-7 cells (upper left side).

The repressor domain consists of two distinct motifs: (i) the SNAG motif, which is conserved in many other proteins including other SNAI family members, Gfi1 and Gfi2, and ZEB1 and ZEB2 (1-3), and (ii) the P-motif, which we found through mutational analyses as essential for the repressor function of human SLUG (Bailey, C. K. and Chaudhuri, G., unpublished data). We replaced the seven amino acids of the P-motif of hSLUG (Fig. 9B) with alanine and the resultant SLUG is functionally inactive (Fig. 10).

5. We further characterized the structure and function of the P-motif of SLUG repressor domain as a unique CtBP1-recruiting site. We found that SLUG recruits a co-repressor, CtBP1, which in turn recruits HDAC1, and that leads to gene repression by chromatin remodeling via histone deacetylation. We have shown by chromatin immunopreciptation assays that SLUG co-localizes with the co-repressor protein CtBP1 when it is bound to the promoters of BRCA2, claudin 7, UBE2D3, VDR and dynactin 5 genes (see Figs. 11 and 12). On the other hand, yeast two-hybrid and *in vitro* co-immunoprecipitation analyses showed that human SLUG does not bind strongly with human CtBP1 (11).

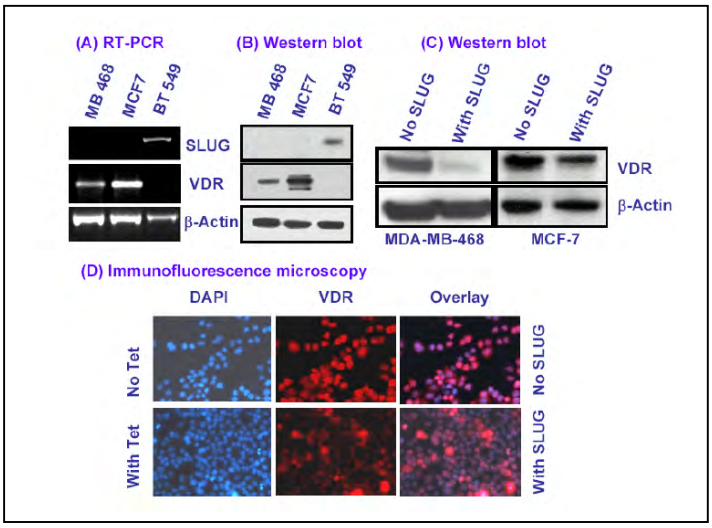


Fig. 6. Down regulation of VDR in SLUG-expressing human breast cells. RT-PCR (A) and Western blotting (B) analyses data showing the expressions of SLUG and VDR mRNA and protein, respectively, in different human breast cancer cells. (C) Western blotting analysis data showing tetracycline-inducible repression of VDR protein levels in the recombinant MCF7 and MDA-MB-468 cells. (D) Immunoflorescence analysis showing tetracycline-inducible repression of VDR protein levels in the recombinant MDA-MB-468 cells. Left panel, the nuclei of the cells were stained with DAPI (blue); middle panel, VDR protein was tagged with red Alexafluor dye; and, right panel, the superimposed photograph. β-actin mRNA and protein were used as loading controls in these studies.

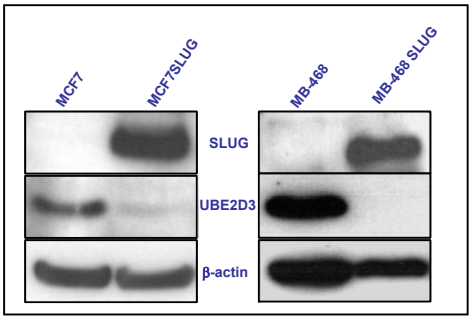


Fig. 7. Down regulation of UBE2D3 in SLUG-expressing human breast cells. Western blotting analysis data showing tetracycline-inducible repression of UBE2D3 protein levels in the recombinant MCF7 and MDA-MB-468 cells. β-actin mRNA and protein were used as loading controls in these studies.

Swapping of the SLUG P-domain with the P-domain from another CtBP1-binding protein ZEB1, led to the binding of the recombinant SLUG strongly with CtBP1 in the yeast two-hybrid system (Figs. 13-15). Thus, CtBP1 may be indirectly recruited to the SLUG-containing silencing complex by an adapter protein. We are currently working on the identification of such proteins in the complex by chromatin immunopull down and proteomics analysis. This study will help us to understand the mode of action of SLUG in human breast cells and to develop peptide aptamers to prevent SLUG gene functions in human breast cancer cells.

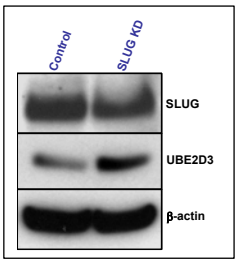


Fig. 8. Down regulation of UBE2D3 in SLUG-expressing human breast cells. Western blotting analysis data showing tetracycline-inducible repression of UBE2D3 protein levels in the recombinant MCF7 and MDA-MB-468 cells. β-actin mRNA and protein were used as loading controls in these studies.

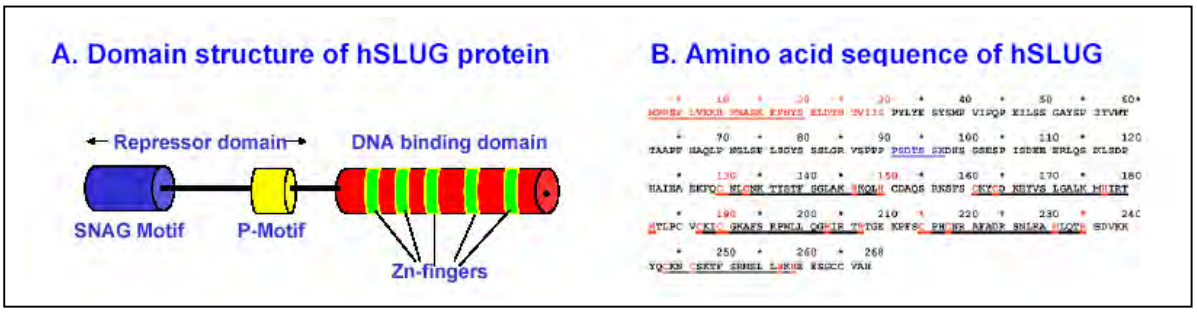
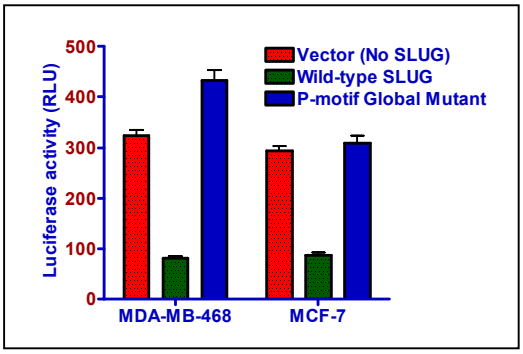


Fig. 9. (A) Domain structure of hSLUG protein. (B) Amino acid sequences of hSLUG protein showing different functional domains. The SNAG motif (residues 1-20), the P-motif (residues 91-97) and the five zinc finger motifs (residues 130-150, 161-181, 187-207, 215-235, and 243-259) are underscored.

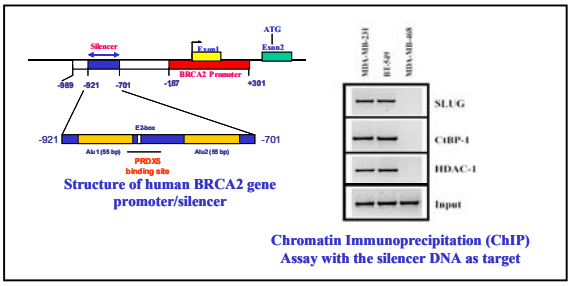
KEY RESEARCH ACCOMPLISHMENTS



(i) Employing the ChIP-DSL techniques (Aviva Systems Biology, San Diego, CA), we have identified 154 genes in the human breast cells that are tightly binding to the transcriptional silencer protein SLUG at the E2-boxes of their promoters.

(ii) The gene promoters we are following up include those of claudin 7, VDR, UBE2D3 and dynactin 5.

Fig. 10: Effect of global mutation of P-motif in hSLUG on the repressor activity of SLUG in human SLUG-negative breast cells. PSDTSSK sequence at the P-motif of human SLUG protein was changed by GeneSOEing to AAAAAAA. We called this mutant SLUG as P-motif global mutant. We also have made point mutants replacing one amino acid at a time with alanine and testing currently their repressor functions.



(iii) By mutational analysis, we identified two distinct motifs in the repressor domain of the SLUG protein as essential for the repressor function of SLUG.

Fig. 11: Binding of SLUG, CtBP-1 and HDAC-1 to the BRCA2 silencer DNA in the nucleus of the SLUG-expressing cells *in vivo*.

(iv) We further characterized the structure and function of the P-motif of SLUG repressor domain as a unique CtBP1-recruiting site.

REPORTABLE OUTCOMES: The research performed directly or indirectly contributed to the following publications and poster abstracts.

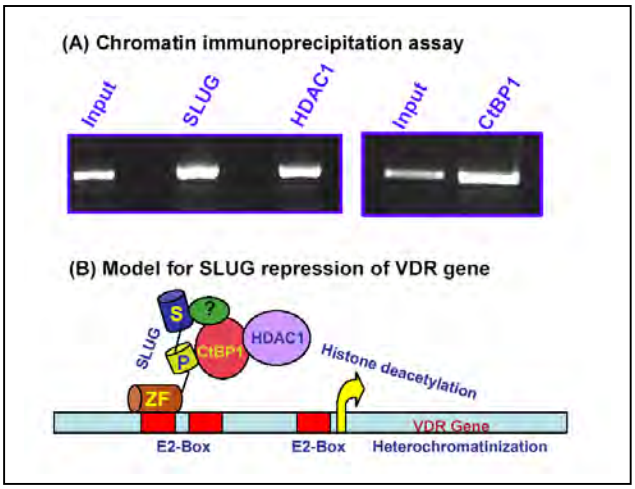


Fig. 12. *In vivo* binding of SLUG, CtBP1 and HDAC1 to the VDR promoter in human breast cells. (A) ChIP analysis data showing the *in vivo* binding of hSLUG, CtBP1, and HDAC1 at the native VDR gene promoter in the lentivirus-transformed MDA-MB-468 cells. The expression of hSLUG was induced with tetracycline (1 μg/ml) for 48 h before the ChIP analysis. Anti-FLAG antibody was used to pull down the SLUG complex. (B) Model showing the possible mode of action of hSLUG to repress VDR gene expression in human breast cells. For simplicity, hSLUG binding to only one of the three E2-boxes is shown. The hypothetical protein, denoted with '?', is proposed to help recruit CtBP1 at the P-motif of hSLUG. ZF, zinc finger of hSLUG; P, the P-motif; and S, the SNAG motif of hSLUG.

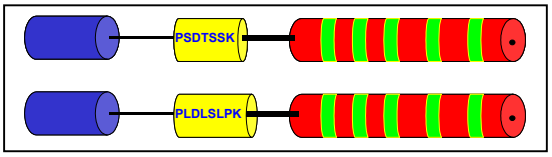


Fig. 13: The CtBP1 domain swap (d.s.) mutant of human SLUG protein. The PSDTSSK sequence was replaced (swapped) by the consensus CtBP1 binding site in ZEB1 protein (PLDLSLPK).

Publication:

1. Bailey, C. K., Misra, S., Mittal, M. K., and **Chaudhuri, G**. (2007) Human SLUG does not directly bind to CtBP1. ***Biochem. Biophys. Res. Commun***. **353**, 661-664.

Mittal, M., Myers, J. N., Misra, S., Bailey, C. K. and Chaudhuri, G. (2008) *In vivo* binding to and functional repression of the VDR gene promoter by SLUG in human breast cells. ***Biochem. Biophys. Res. Commun***. **(In Press)**

Meeting abstracts:

1. Mittal, M. K., Bailey, C. K., Misra, S. and **Chaudhuri, G.** (2006) SLUG-dependent modulation of Claudin 7 gene expression in metastatic human breast tumor cells. **Presented as a poster at the ASBMB Transcription Meeting at Kiawah Island Resort in SC on Nov 2-6, 2006**.

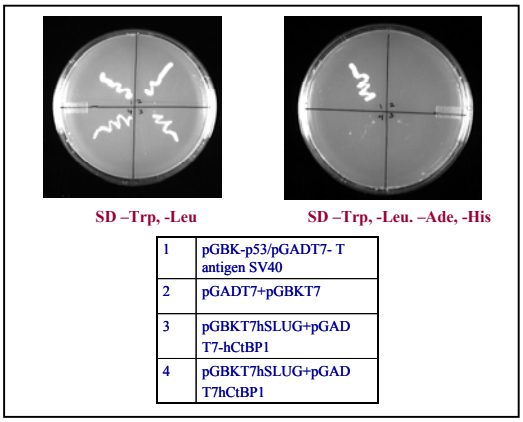


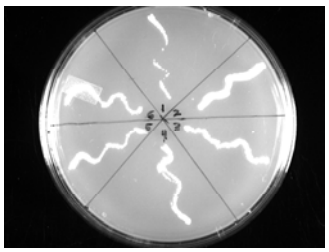
Fig. 14: Evaluation of potential interactions of hSLUG with hCtBP1 by yeast-2-hybrid analysis. Native SLUG protein failed to interact with CtBP1 (11).

2. Mittal, M. K., Bailey, C. K., Myers, J., Misra, S. and **Chaudhuri, G.** (2007) Differential contribution of distinct E2-box elements in the dual regulation of human claudin 7 gene promoter by SNAIL and SLUG in human breast cells. **Presented as a poster in the Annual Meeting of American Association for Cancer Research in Los Angeles, CA, April 14-18, 2007.**

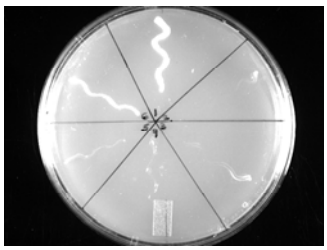
3. Bailey, C. K., Misra, S., Mittal, M. K., and **Chaudhuri, G.** (2007) Human SLUG does not directly bind to CtBP1. **Presented as a poster in the Annual Meeting of American Association for Cancer Research in Los Angeles, CA, April 14-18, 2007.**

We will be presenting the following posters at the Vanderbilt-Ingram Cancer Center RETREAT – 2008, *Genomic Instability in Cancer,* May 6, 2008

1. Mittal, Mukul K., Bailey, Charvann K., and Chaudhuri, Gautam (2008) Repression of the Cyclin D1 and P53 regulatory ubiquitin–conjugating enzyme UBE2D3 by SLUG in human breast cells.
2. Bailey, Charvann K., Misra, Smita, Mittal, Mukul K., and Chaudhuri, Gautam (2008) Internal P-domain of human SLUG protein is critical for co-repressor recruitment and its repressor activity in breast cancer cells.
3. Myers, Jeremy N., Mittal, Mukul K., Misra, Smita, Bailey, Charvann K., and Chaudhuri, Gautam (2008) *In vivo* binding to and functional repression of the VDR gene promoter by SLUG in human breast cells.



SD –Trp, -Leu



SD –Trp, Leu, -His -Ade

1	pGAD SV40 T antigen +pGBK-p53
2	pGADT7+ PGBKT7
3	pGADT7+pGBK-CtBP1 domain swap
4	pGAD-hCtBP1+pGBKT7
5	pGADT7-CtBP1+ pGBKT7- WT-hSLUG
6	pGADT7-CtBP1+ pGBK-CtBP1 domain swap

Fig. 15: Evaluation of potential interactions of the CtBP1 domain swap mutant with hCtBP1 by yeast 2-hybrid analysis. The CtBP1 domain swap mutant of human SLUG was able to interact directly with human CtBP1 protein in the yeast 2-hybrid analysis.

We will be presenting the following posters at the Department of Defense (DOD) Breast Cancer Research Program (BCRP) Era of Hope 2008 Meeting, Baltimore Convention Center, Wednesday, June 25, 2008 - Saturday, June 28, 2008

1. Mittal, M. K. and Chaudhuri, G. (2008) ChIP-DSL technology reveals an extensive SLUG-binding program on human gene promoters in breast cells.
2. Tripathi, M. K., Misra, S. and Chaudhuri, G. (2008) Mechanisms of silencing and desilencing of BRCA2 gene expression in human breast cells.

CONCLUSION: Employing the ChIP-DSL techniques, we have identified 154 genes in the human breast cells that are tightly binding to the transcriptional silencer protein SLUG at the E2-boxes of their promoters.The gene promoters we are following up include those of claudin 7, VDR, UBE2D3 and dynactin 5.By mutational analysis, we identified two distinct motifs in the

repressor domain of the SLUG protein as essential for the repressor function of SLUG. We further characterized the structure and function of the P-motif of SLUG repressor domain as a unique CtBP1-recruiting site.

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APPENDICES: One new preprint and five new abstracts are attached. The others were included in the previous report.

Elsevier Editorial System(tm) for Biochemical and Biophysical Research Communications
Manuscript Draft

Manuscript Number:

Title: In vivo binding to and functional repression of the VDR gene promoter by SLUG in human breast cells

Article Type: Regular Article

Keywords: SLUG; VDR; E2-box, transcriptional repression; CtBP1; HDAC1

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MEHARRY
MEDICAL COLLEGE

SCHOOL OF MEDICINE
Department of Microbial Pathogenesis & Immune Response

April 24, 2008

The Editor
BBRC

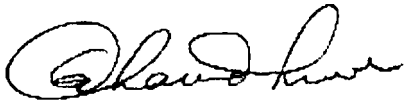
Dear Editor:

We are submitting the manuscript "***In vivo* binding to and functional repression of the VDR gene promoter by SLUG in human breast cells**" to be considered for publication in the BBRC.

We report here the *in vivo* binding of the transcriptional repressor SLUG to the VDR gene promoter in human breast cell nucleus and the inhibition of VDR gene expression by chromatin remodeling induced by SLUG.

We are not suggesting any names of potential reviewers for this manuscript. We request that the associate editor to make this decision.

Looking forward to hear from you soon.



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In vivo binding to and functional repression of the VDR gene promoter by SLUG in human breast cells

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Running Title: SLUG inhibition of VDR gene expression

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Abstract

The regulation of vitamin D receptor (VDR), a key mediator in the vitamin D pathway, in breast cancer etiology has long been of interest. We have shown here that the transcriptional repressor protein SLUG inhibits the expression of VDR in human breast cancer cells. To explore the possibility that SLUG regulates the *VDR* gene promoter, we cloned a 628 bp fragment (–613 to +15) of the human *VDR* gene promoter. This region contains three E2-box sequences (CAGGTG/CACCTG), the classical binding site of SLUG. SLUG specifically inhibited *VDR* gene promoter activity. Chromatin-immunoprecipitation (ChIP) assays revealed that SLUG is recruited on the native *VDR* promoter along with the co-repressor protein CtBP1 and the effector protein HDAC1. These data suggests that SLUG binds to the E2-box sequences of the VDR gene promoter and recruits CtBP1 and HDAC1, which results in the inhibition of VDR gene expression by chromatin remodeling.

Keywords: SLUG, VDR, E2-box, transcriptional repression, CtBP1, HDAC1.

Introduction

The vitamin D receptor (VDR) [1-3] is a ligand-regulated transcription factor that mediates most biological effects of 1,25-dihydroxyvitamin D (VD). *In vitro* studies have shown that the VDR ligand, VD, modulates key proteins involved in signaling, proliferation, differentiation, and survival of normal mammary epithelial cells. However, many transformed breast cancer cells lose sensitivity to VD owing to down regulation of VDR function [1-3].

SLUG is a member of the SNAI family of C_2H_2-zinc finger family of transcriptional repressors [4-6]. It is involved in the epithelial-mesenchymal transition during development [5], acts as an inhibitor of apoptosis [7], and causes tubulogenesis during breast and kidney developments [4, 5]. The genes inhibited by SLUG include E-cadherin [8], claudins [9], BRCA2 [10], and cytokeratins [11]. Our ChIP-DSL analysis of 20,000 human gene promoter array revealed that more than 150 promoters bind to SLUG at their promoters (Mittal, M.K. and Chaudhuri, G., unpublished data). VDR gene is one of the candidate SLUG-regulated genes. Here, we report that SLUG indeed binds *in vivo* to the VDR gene promoter in human breast cell nucleus and inhibits VDR gene expression by chromatin remodeling.

Materials and methods

Cell culture and reagents

Human breast cancer cells MCF7, MDA-MB-468, MDA-MB-231 and BT549 were obtained from ATCC (Manassas, VA) and were cultured in ATCC-recommended media [10, 11]. FLAG M2 antibody was purchased from Sigma Chemical Co. (St. Louis, MO). CtBP1 and HDAC1 antibodies were purchased from UPSTATE Millipore (Burlington, MA). VDR and SLUG (G18) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Generation of stable clones

Human SLUG (hSLUG) gene ORF (NM_003068) was PCR amplified [10] from the cDNAs derived from MDA-MB-231 cells with Cla I and Bam HI site-containing

primers (5´-CAAGGTACCATGCCGCGCTCCTTCCTGC-3´, and 5´-CAAGGATCCGTGT GCTACACAGCAGCC-3´). The reverse primer did not have the endogenous stop codon. The PCR product was cloned at the Cla I/Bam HI sites of p3XFLAG-CMV-14 vector (Sigma). The SLUG-3xFLAG sequences were then amplified with 5´-CACCATGCCGCGCTCCTTCCT-3´ and 5'-ATCACTACTTGTCATCGTCATCCTTGTAG TCG-3´ primers to clone directionally into the Gateway entry vector pENTR/D-TOPO (Invitrogen, Carlsbad, CA). The SLUG-3xFLAG ORF was then transferred to pLenti4/TO/V5-DEST vector (Invitrogen) by recombination using Gateway cloning reagents and protocols (Invitrogen). Transfection of 293 FT packaging cells with the plasmid constructs and human breast cells with the virus were done as described before [10]. The blasticidin (25 µg/ml)-resistant tetracycline repressor-expressing cells were further transduced by pLenti4/TO/V5-SLUG-3xFLAG-containing virus as described above, and the stable cell line was selected with 250 µg/ml zeocin. These double-resistant cell lines were maintained in blasticidin and zeocin. Expression of SLUG was induced by tetracyclin (1 µg/ml) for 24-48 h.

Immunofluorescence analysis

Cells were cultured in 8-well chamber slides for 24 h in complete media, washed with PBS, fixed and permeabilized with ice-cold methanol for 10 min. After blocking in 5% goat serum in PBS, the cells were incubated with the primary antibody (Santa Cruz Biotechnology) followed by secondary antibody conjugated with the red fluorescent dye (ALexa Fluor R555-conjugated donkey anti mouse IgG, Invitrogen). The cells were subsequently stained with DAPI (Sigma). Finally, each slide was examined by

fluorescence microscopy in a Nikon TE2000-E inverted wide-field microscope. Each representative image was examined and digitally recorded at the same cellular level and magnification.

RT–PCR analysis

RNA was extracted from cells using Trizol reagent (Invitrogen) and PCR amplifications were performed as described [10]. Primers used were: for SLUG, 5´-ATGCCGCGCTCCTTCCTGC-3´ and 5'-ATGGAGGAGGGGGACTCACTCG-3´, for VDR, 5´-CCAGTTCGTGTGAATGATGG-3´ and 5´-AGATTGGAGAAGCTGGACGA-3´, for β-actin, 5´-GCTCGTCGTCGACAACGGCTC-3´ and 5´-CAAACATGATCTG GGTCATCTTCTC-3´.

Luciferase reporter assay

We PCR amplified human VDR gene promoter (–613 to +15, NM_000376) from total DNA isolated from BT549 cells with 5´-GCTGCCAAGGTGATATCGGG-3´ and 5´-CGCTGCCGCCTTTTGACAAG-3´ primers. The amplified DNA was cloned into the pCR-II-TOPO vector (Invitrogen) and subsequently subcloned into the Eco RI site of pRL-Null vector (Promega). Cells were seeded on 24-well tissue culture plates and post 24 h, they were transfected with pGL3-Control and pRL-*VDR* promoter construct using Lipofectamine 2000 transfection reagent (Invitrogen). Luciferase activity was assayed 48 h later using a dual luciferase assay kit (Promega), as described before [10].

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assays were performed as described previously [10]. Immunoprecipitations were performed using FLAG (for SLUG), CtBP1 or HDAC1 antibodies. VDR promoter DNA was amplified from the ChIP DNA using the primers described above.

Immunoblot analysis

Cells from stable clones were grown in complete medium. Protein extracts were made and Western blotting was performed as described [10]. Cell lysates containing equal amounts of protein were resolved by 4-12% SDS-PAGE, transferred to nitrocellulose membranes, probed with the appropriate antibodies, and detected by means of enhanced chemiluminescence [10].

Results and Discussion

Inducible expression of FLAG-tagged SLUG in the SLUG-negative MDA-MB-468 and MCF-7 cells.

Human SLUG protein is 268 amino acids long with two functional domains: (i) the N-terminal repressor domain, and (ii) the C-terminal DNA binding domain (Fig. 1A and 1B). The DNA binding domain has five C_2H_2 type zinc fingers, which are essential for the interaction of this repressor protein with the E2-box sequences (CAGGTG/CACCTG) at the promoters of SLUG-target genes [4-6]. The repressor domain consists of two distinct motifs: (i) the SNAG motif, which is conserved in many other proteins including other SNAI family members, Gfi1 and Gfi2, and ZEB1 and

ZEB2 [4-6], and (ii) the P-motif, which we found through mutational analyses as essential for the repressor function of human SLUG (Bailey, C. K. and Chaudhuri, G., unpublished data). We replaced the seven amino acids of the P-motif of hSLUG (Fig. 1B) with alanine and used the resultant functionally inactive SLUG as a control in the studies described here.

To study the binding of SLUG to its target gene promoters, we expressed recombinant SLUG in SLUG-negative human breast cells *e.g*., MCF-7 and MDA-MB-468 [10]. Attaching the 3xFLAG epitope at the C-terminus of hSLUG protein did not alter the repressor activity of this protein. We employed a lentiviral vector system to express recombinant SLUG from a tetracycline-inducible promoter. Figs. 1C and 1D shows that the SLUG mRNA and the 3XFLAG-tagged SLUG protein are abundantly expressed both in the recombinant MDA-MB-468 and MCF-7 cells in a tetracycline-inducible manner. These recombinant cells inducibly expressing functionally active 3xFLAG-tagged hSLUG were used for the studies described below.

Repression of VDR gene expression by hSLUG in MDA-MB-468 and MCF-7 cells.

While studying SLUG-binding gene promoters in human breast cells using the 3xFLAG-SLUG-expressing MDA-MB-468 and MCF-7 cells employing the ChIP-DSL technique [12] with the reagents and human 20,000 gene promoter chip from Aviva Systems Biology (San Diego, CA), we discovered that VDR gene promoter binds strongly with the SLUG protein. Since VDR and SLUG proteins are relevant in human breast cancer etiology, we characterized further the interactions of SLUG and the VDR gene promoter in the human breast cells. With few cultured human breast cells, we

found that there is an inverse relationship between SLUG and VDR gene expressions. The noninvasive MDA-MB-468 and MCF-7 cells do not express SLUG gene and they have significant levels of VDR mRNA and protein (Fig. 2A and 2B). Whereas, the highly invasive BT549 cells expresses SLUG but no VDR (Fig. 2A and 2B). When we induced the expression of SLUG in the recombinant MDA-MB-468 and MCF-7 cells, the levels of the VDR protein decreased significantly (Fig. 2C). Expression of non-functional SLUG protein did not cause any such effect on the VDR protein levels (data not shown). Our immunofluorescence microscopy data further verified the down regulation of VDR gene expression in the presence of SLUG in the recombinant MDA-MB-468 cells (Fig. 2D). These data strongly suggests that SLUG inhibits the expression of VDR gene in human breast cells.

Inhibition of VDR gene promoter activity by hSLUG

We then evaluated whether hSLUG can inhibit the cloned VDR gene promoter. We amplified a 628 bp (–613 to +15) promoter sequence (Fig. 3A) from human VDR gene and cloned that in front of *Renilla* luciferase gene in pRL-Null vector. This promoter sequence has three E2-boxes at the upstream of the transcription start site (Fig. 3A). Tetracycline induction of hSLUG expression in recombinant MDA-MB-468 and MCF-7 cells showed down regulation of VDR gene promoter activities (Fig. 3B). These data suggest that hSLUG works through the E2-box containing minimal promoter sequence of human VDR gene to repress it.

In vivo binding of SLUG, CtBP1 and HDAC1 proteins to the VDR gene promoter in human breast cells

To test whether SLUG is indeed binds to VDR gene promoter in the breast cell nuclei and to validate the co-repressor and effector requirements in this repression process, we performed ChIP analysis with antibodies against FLAG, CtBP1 and HDAC1 in the recombinant MDA-MB-468 cells. We previously found that CtBP1 is the co-repressor for SLUG-repression of human BRCA2 gene [10]. We also detected HDAC1 as the effector for the heterochromatinization of human BRCA2 gene promoter [10]. Fig. 4A shows that VDR promoter indeed binds to SLUG in the nucleus of the recombinant MDA-MB-468 cells. CtBP1 and HDAC1 also bound to the VDR promoter when SLUG was expressed (Fig. 4A). These bindings were dependent on tetracycline induction of SLUG (data not shown), suggesting that SLUG binding to the VDR promoter is a prerequisite for CtBP1 and HDAC1 binding to this promoter. Non-functional P-motif mutated SLUG also could not recruit CtBP1 or HDAC1 to the VDR gene promoter (data not shown). Based on these observations, we propose a model for SLUG-mediated down regulation of human VDR gene expression by chromatin remodeling (Fig. 4B). According to this model, SLUG is recruited to any or all three of the E2-box sequences at the VDR gene promoter through its DNA binding domain. We have shown previously that hSLUG cannot directly bind to CtBP1 [13]. Thus, assisted perhaps by other transcription regulator(s), CtBP1 is recruited at the P-motif of SLUG. CtBP1 then recruits HDAC1, and perhaps other effectors (e.g., HMT1), to catalyze histone modification and silencing of the VDR gene promoter. Although VDR gene promoter was shown to be regulated by other E2-box binding proteins in non-breast cells [14, 15],

ours is the first report of the direct involvement of SLUG in the modulation of the expression of VDR gene in human breast cells.

Abbreviations

VDR: Vitamin D receptor; VD: Vitamin D, CtBP1: C-terminal binding protein 1; HDAC1: Histone deacetylase 1; HMT1: Histone methyl transferase 1.

Acknowledgements

This work was supported by the DOD-CDMRP IDEA Grant# W81XWH-06-1-0466 to GC. Immunofluorescence analysis was performed with the help of Dr. S. Goodwin, Director, MMC Morphology Core (supported by NIH grants U54NS041071-06, G12RR03032-19, U54CA91408**,** and U54RR019192-04).

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Figure Legends

Fig. 1. Inducible expression of FLAG-tagged SLUG in MCF7 and MDA-MB-468 cells. (A) Domain structure of hSLUG protein. (B) Amino acid sequences of hSLUG protein showing different functional domains. The SNAG motif (residues 1-20), the P-motif (residues 91-97) and the five zinc finger motifs (residues 130-150, 161-181, 187-207, 215-235, and 243-259) are underscored. RT-PCR (C) and Western blotting (D) analyses data showing tetracycline-inducible expression of hSLUG mRNA and protein, respectively. β-actin mRNA and protein were used as loading controls in these studies.

Fig. 2. Down regulation of VDR in SLUG-expressing human breast cells. RT-PCR (A) and Western blotting (B) analyses data showing the expressions of SLUG and VDR mRNA and protein, respectively, in different human breast cancer cells. (C) Western blotting analysis data showing tetracycline-inducible repression of VDR protein levels in the recombinant MCF7 and MDA-MB-468 cells. (D) Immunoflorescence analysis showing tetracycline-inducible repression of VDR protein levels in the recombinant MDA-MB-468 cells. Left panel, the nuclei of the cells were stained with DAPI (blue); middle panel, VDR protein was tagged with red Alexafluor dye; and, right panel, the superimposed photograph. β-actin mRNA and protein were used as loading controls in these studies.

Fig. 3. Negative regulation of VDR gene promoter activity in SLUG-expressing human breast cells. (A) Nucleotide sequence of human VDR gene promoter showing

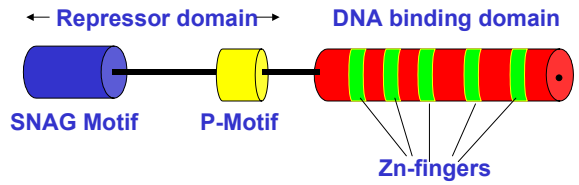
(underscored) the SLUG-binding E2-box (CAGGTG/CACCTG) elements. The upstream sequences are shown in lower case letters. The 5´-end of the exon 1 sequences is in uppercase letters. (B) Dual luciferase assay data showing the repression of the function of VDR gene promoter in the recombinant MCF7 and MDA-MB-468 cells. Results are mean ± SE (n=6). The differences in the luciferase activities between the control and the tetracycline-induced cells were statistically significant ($p<0.001$).

Fig. 4. *In vivo* binding of SLUG, CtBP1 and HDAC1 to the VDR promoter in human breast cells. (A) ChIP analysis data showing the *in vivo* binding of hSLUG, CtBP1, and HDAC1 at the native VDR gene promoter in the lentivirus-transformed MDA-MB-468 cells. The expression of hSLUG was induced with tetracycline (1 μg/ml) for 48 h before the ChIP analysis. Anti-FLAG antibody was used to pull down the SLUG complex. (B) Model showing the possible mode of action of hSLUG to repress VDR gene expression in human breast cells. For simplicity, hSLUG binding to only one of the three E2-boxes is shown. The hypothetical protein, denoted with '?', is proposed to help recruit CtBP1 at the P-motif of hSLUG. ZF, zinc finger of hSLUG; P, the P-motif; and S, the SNAG motif of hSLUG.

Figure 1

FIG.1. Mittal *et al.*, VDR

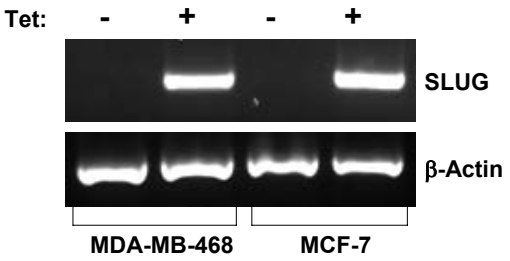
A. Domain structure of hSLUG protein



B. Amino acid sequence of hSLUG

```
    *    10   *    20   *    30   *    40   *    50   *    60*
MPRSF LVKKH FNASK KPNYS ELDTH TVIIS PYLYE SYSMP VIPQP EILSS GAYSP ITVWT

    *    70   *    80   *    90   *   100   *   110   *   120
TAAPF HAQLP NGLSP LSGYS SSLGR VSPPP PSDTS SKDHS GSESP ISDEE ERLQS KLSDP

    *   130   *   140   *   150   *   160   *   170   *   180
HAIEA EKFQC NLCNK TYSTF SGLAK HKQLH CDAQS RKSFS CKYCD KEYVS LGALK MHIRT

    *   190   *   200   *   210   *   220   *   230   *   240
HTLPC VCKIC GKAFS RPWLL QGHIR THTGE KPFSC PHCNR AFADR SNLRA HLQTH SDVKK

    *   250   *   260   *  268
YQCKN CSKTF SRMSL LHKHE ESGCC VAH
```

C. RT-PCR analysis



D. Western blotting

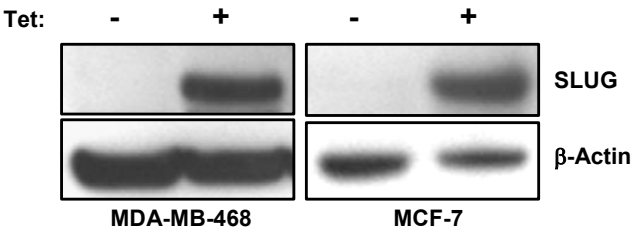


Figure 2

FIG.2. Mittal *et al.*, VDR

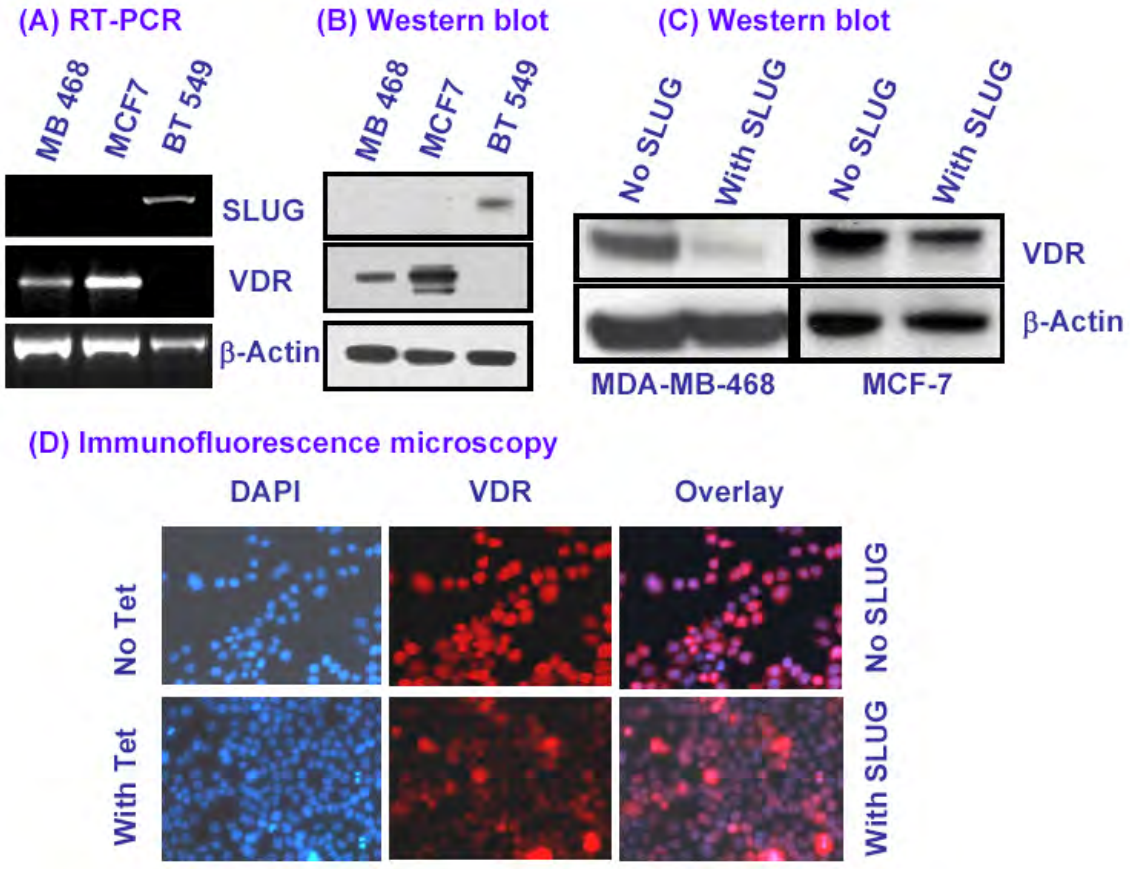


Figure 3

FIG. 3. Mittal *et al.*, VDR

(A) VDR gene promoter

-613
gctgccaaggtgatatcgggtgggagcaatga
cgcaactccggtttccacttcggccccccggg
atattttaccctaatctgtgggatcaggctga
gcttcctggcgttctgcagcagtaacaggttg
gcgagcggagcccgggatttcccattcgtgcg
gagctagccgccggtgccagtcggcaggcgcc
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aagcgtgccttgccctatggacgacggtcgat
gaaaatttcacgagttagagtatctaaggcta
cagcgtggcctatagggtggttgattccaagt
caagatggttgcagcgccaacggagctcctgg
caagagaggactggacctgtgggcggggcgga
ggggcggggcggggccggggcggggcctgacc
gagaggcggggccaggtgctgggctgtctctg
cttgtCAAAAGGCGGCAGCG +15

(B) VDR promoter activity

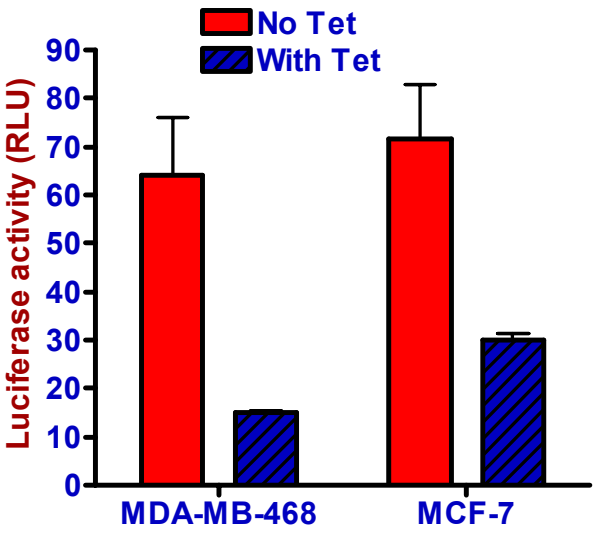
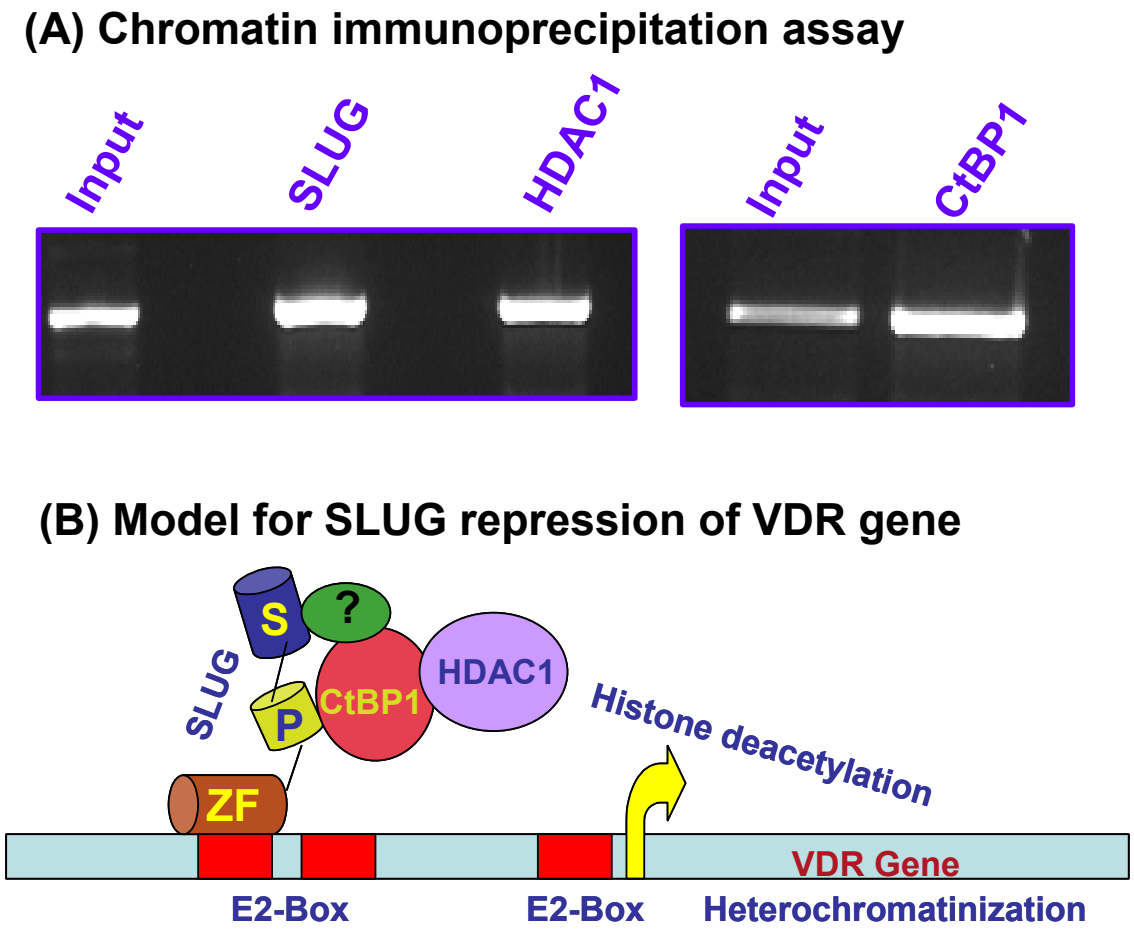


Figure 4

FIG. 4. Mittal *el al.*, VDR



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Title of Abstract: Repression of the Cyclin D1 and P53 regulatory ubiquitin–conjugating enzyme UbE2D3 by SLUG in human breast cells

Repression of the Cyclin D1 and P53 regulatory ubiquitin–conjugating enzyme UBE2D3 by SLUG in human breast cells

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SLUG is a transcriptional repressor protein implicated in the proliferation and metastasis of several human cancer cells. It binds to the E2-box sequence of its target gene promoters and down regulates their expressions by chromatin remodeling. While studying SLUG-binding gene promoters in the human breast cells by ChIP-DSL techniques, we found the Cyclin D1 and P53 regulator ubiquitin ligase gene UbE2D3 as one of them. The E2 enzyme UBE2D3 works with the E3 enzymes $SCF^{FBX4\text{-}aB\ crystalline}$ and MDM2, respectively, to polyubiquitinylate cyclin D1 and P53 proteins. Polyubiquitinylated cyclin D1 and P53 are then degraded by 26S proteasomes. Down regulation of UBE2D3 prevents the polyubiquitinylation process resulting in increase of the levels of these proteins. We over expressed SLUG in the SLUG-negative MCF7 and MDA-MB-468 cells from a doxycycline inducible promoter. SLUG over expression down regulated the mRNA and protein levels of UBE2D3 in these cells. siRNA-mediated knock down of SLUG in the SLUG positive MDA-MB-231 cells elevated the mRNA and protein levels of UBE2D3 in these cells The activity of the cloned UBE2D3 gene promoter was also down regulated in the cells expressing functional SLUG repressor protein. Mutational analysis identified the E2-box sequence at the UBE2D3 gene promoter that is responsible for SLUG-mediated repression. Chromatin immunoprecipitation data revealed co-recruitment of CtBP1 and HDAC1 at the UbE2D3 gene promoter in the SLUG-expressing cells further indicating that SLUG represses this gene through chromatin remodeling. siRNA-mediated knock down of UBE2D3 in the SLUG-negative human breast cells elevated the levels of cyclin D1 and invasiveness of breast cancer cells. Cyclin D1 is a molecular target for germ cell tumor growth, maturation, and chemotherapy response. Our study indicates a novel mechanism of up regulation of cyclin D1 levels in the cells by a tumor promoter transcription factor SLUG. A single E2 enzyme such as UBE2D3 can usually interact with several E3 ubiquitin ligases and thereby affect multiple targets. Thus, proteins other than cyclin D1 and P53 could also act as downstream targets of UBE2D3. Supported by the DOD-CDMRP IDEA Grant# W81XWH-06-1-0466 and the Susan G. Komen Breast Cancer Foundation grant# BCTR0707627 to GC.

Please return this abstract registration form to Mary Ann Stevens in the VICC Administrative Office [697 PRB (6838); fax, (93)6-6865; email, maryann.stevens@vanderbilt.edu] *NO LATER THAN* April 18, 2008.
If you have any questions, do not hesitate to contact Mary Ann Stevens at 6-1863, or the email address above.

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Title of Abstract: Internal P-domain of human SLUG protein is critical for co-repressor recruitment and its repressor activity in breast cancer cells

Internal P-domain of human SLUG protein is critical for co-repressor recruitment and its repressor activity in breast cancer cells

Charvann K. Bailey, Smita Misra, Mukul K. Mittal and Gautam Chaudhuri

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SLUG is a transcriptional repressor protein implicated to have a major role in the oncogenesis and metastasis of human breast cells. Over expression of SLUG protein in epithelial cells leads to loss of expression of key cell-cell adhesion molecules, such as E-cadherin, claudins, and occludin. SLUG also down regulates the tumor suppressor protein BRCA2 in human breast cells. SLUG contains a highly conserved region at the C terminus of the protein containing 4–6 zinc fingers of the C_2H_2 type. The zinc fingers mediate sequence-specific interactions with DNA. The N terminus of SLUG contains the evolutionarily conserved SNAG (for Snail/Gfi) domain that was thought to mediate the transcriptional repression function of SLUG. We found that SLUG recruits a co-repressor, CtBP1, which in turn recruits HDAC1, and that leads to gene repression by chromatin remodeling via histone deacetylation. We have shown by chromatin immunopreciptation assays that SLUG co-localizes with the co-repressor protein CtBP1 when it is bound to the promoters of BRCA2, claudin 7, UBE2D3, VDR and dynactin5 genes. Alanine replacement mutagenesis of the canonical CtBP1 binding sequence in the SLUG protein (the P-domain) followed by reporter gene expression analysis reveled that this sequence is essential for the repressor function of SLUG. On the other hand, yeast two-hybrid and *in vitro* co-immunoprecipitation analyses showed that human SLUG does not bind strongly with human CtBP1. Swapping of the SLUG P-domain with the P-domain from another CtBP1-binding protein ZEB1, led to the binding of the recombinant SLUG strongly with CtBP1 in the yeast two-hybrid system. Thus, CtBP1 may be indirectly recruited to the SLUG-containing silencing complex by an adapter protein. We are currently working on the identification of such proteins in the complex by chromatin immunopull down and proteomics analysis. This study will help us to understand the mode of action of SLUG in human breast cells and to develop peptide aptamers to prevent SLUG gene functions in human breast cancer cells.
Supported by the DOD-CDMRP IDEA Grant# W81XWH-06-1-0466 and the Susan G. Komen Breast Cancer Foundation grant# BCTR0707627 to GC.

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Title of Abstract: ***In vivo* binding to and functional repression of the VDR gene promoter by SLUG in human breast cells**

***In vivo* binding to and functional repression of the VDR gene promoter by SLUG in human breast cells**

Jeremy N. Myers, Mukul K. Mittal, Smita Misra, Charvann K. Bailey and Gautam Chaudhuri

The regulation of vitamin D receptor (VDR), a key mediator in the vitamin D pathway, in breast cancer etiology has long been of interest. Anti-proliferative and pro-differentiating effects of vitamin D have been observed in VDR-positive breast cancer cells, indicating that transformation per se does not abolish vitamin D signaling. However, many transformed breast cancer cells lose sensitivity to vitamin D secondary to down regulation of VDR function. SLUG is a member of the SNAI family of C_2H_2-zinc finger family of transcriptional repressors. It is involved in the epithelial-mesenchymal transition during development, acts as an inhibitor of apoptosis, and causes tubulogenesis during breast and kidney developments. The genes inhibited by SLUG include cell adhesion molecules (E-cadherin and claudins), BRCA2, and cytokeratins. To identify the genes regulated by SLUG in human breast cells, we expressed FLAG-tagged SLUG from a doxycycline-inducible promoter using a lentiviral vector in the SLUG-negative MCF-7 and MDA-MB-468 cells. Our ChIP-on-chip (ChIP-DSL) analysis of 20,000 human gene promoters revealed that more than 150 genes bind to SLUG at their promoters. VDR gene is one of the candidate SLUG-regulated genes. We have shown here that functional SLUG, but not an inactive mutant SLUG, inhibits the expressions of VDR gene in human breast cancer cells. To explore the possibility that SLUG regulates the *VDR* gene promoter, we cloned a fragment (nucleotides –600 to +23) of the human *VDR* gene promoter. This region contains three E2-box sequences (CACCTG), the classical binding site of SLUG. SLUG specifically inhibited *VDR* gene promoter activity. Further chromatin-immunoprecipitation (ChIP) assays revealed that SLUG is recruited on the native *VDR* promoter along with the co-repressor protein CtBP1 and the effector protein HDAC1 *in vivo*. These data suggests that SLUG binds to the E2-box sequences of the VDR gene promoter and recruits CtBP1 and HDAC1, which results in the inhibition of VDR gene expression by chromatin remodeling. Supported by the DOD-CDMRP IDEA Grant# W81XWH-06-1-0466 and the Susan G. Komen Breast Cancer Foundation grant# BCTR0707627 to GC.

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